Supplementary Information

Ln_3TCAS_2–polyethyleneimine supramolecular nanogels: A platform for neutron capture therapy and complementary magnetic resonance imaging (Ln = lanthanide, TCAS = thiacalix[4]arene-*p*-tetrasulfonate)

Nanaho Shindo,[1] Ryota Sawamura,[1] Ryunosuke Karashimada,[1] Shan Gao,[2] Yoshikazu Ozawa,[2] Kensuke Osada,[2]* Ichio Aoki,[2] Minoru Suzuki,[3] and Nobuhiko Iki[1]*

[1] *Graduate School of Environmental Studies, Tohoku University, 6-6-07 Aramaki-Aoba, Aoba-ku, Sendai 980-8579, Japan*
[2] *Institute for Quantum Medical Science, National Institutes for Quantum Science and Technology, 4-9-1 Anagawa, Inage, Chiba, Chiba 263-8555, Japan*
[3] *Division of Particle Radiation Oncology, Particle Radiation Oncology Research Center, Institute for Integrated Radiation and Nuclear Science, Kyoto University, 2–1010, Asashiro-nishi, Kumatori-cho, Sennan-gun, Osaka, 590-0494 Japan*

* iki@tohoku.ac.jp, osada.kensuke@qst.go.jp

1. Experimental details

1.1 Materials

Thiacalix[4]arene-*p*-tetrasulfonate (TCAS) was prepared using a method reported in previous studies [1]. The stock solution factor was evaluated by the molar ratio method based on the formation of the $Ni^{II}_4TCAS_2$ complex. Stock solutions of Ni^{II}, Tb^{III}, and Gd^{III} were prepared by dissolving $NiCl_2 \cdot 6H_2O$, $Tb(NO_3)_3 \cdot 6H_2O$ and $Gd(NO_3)_3 \cdot 6H_2O$ (Kanto Chemical Co., Ltd., high-purity reagents), respectively, in nitric acid (HNO_3). The concentration factor was determined using a reliable chelatometry method [2]. Poly(ethyleneimine) (PEI, branched, M_w = 25,000, Sigma Aldrich 408727) was purchased from Merck KGaA, Darmstadt. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was obtained from Dojindo Laboratories, Kumamoto. High-purity nitric acid (Fuji Film Wako Pure Chemical Corporation), hydrochloric acid, sodium hydroxide, and ammonia water (Kanto Chemical Co., Ltd., special grade) were used to adjust the pH. Sodium chloride (Kanto Chemical Co., Ltd., special grade) was used to adjust the ionic strength. Super-special-grade hydrogen peroxide was obtained from Fuji Film Wako Pure Chemical Corporation. Phosphorus pentoxide (P_2O_5) was obtained from Kanto Chemical Co., Ltd. (special grade) and used as a desiccant. The MCF-7 cells were obtained from the Institute of Development, Aging, and Cancer, Tohoku University (Japan). The cell culture medium, RPMI 1640 containing l-glutamate, fetal bovine serum (FBS), penicillin-streptomycin (PS), and 0.25% trypsin-EDTA solution were purchased from Thermo Fisher Scientific Inc. Dulbecco's Phosphate-Buffered Saline without calcium or magnesium, PBS(–), was obtained from Fuji Film Wako Pure Chemical Corporation. For cytotoxicity assay, Cell Counting Kit-8 (CCK-8, Dojindo) was used. For live/dead cell imaging, a CellStain Double Staining Kit (Dojindo) containing 1 mM calcein-AM in DMSO and 1.5 mM propidium iodide (PI) in water was used. For organelle staining, Hoechst 33342 and LysoPrime Green (Dojindo Laboratories) were used. To inhibit endocytosis, methyl-β-cyclodextrin and sucrose (Fujifilm Wako Pure Chemical Corporation) were employed. Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetate gadolinium(III) dihydrogen salt hydrate (Gd-DTPA, 97%, Sigma-Aldrich) was purchased from Merck KGaA. Deionized water prepared using an Elga PURELAB Chorus2+ was used in this study.

1.2 Equipment

The pH measurements were performed using an HM-30V indicator (Toa DKK Corporation) equipped with a GST-5425C glass electrode (Toa DKK Corp.). A ultrasonic homogenizer UH-50 (50 W, SMT Co., Ltd., Tokyo) equipped with a probe MS-3 (3 mm o.d.) was used to disperse the nanogel. Dynamic Light Scattering (DLS) measurements were performed using a nano Partica SZ-100 instrument (HORIBA, Ltd.) with a four-sided transmissive disposable cell and an optical path length of 1.0 cm. For ζ potential measurement, a dedicated carbon electrode cell was used. Centrifugation was performed using an AS185 apparatus (As One Corporation) and a Sorvall ST 8R centrifuge (Thermo Fisher Scientific, Inc.). Elemental analyses were performed using an iCap 6500 ICP-AES instrument (Thermo Scientific Inc.) or an Agilent 8800 Triple Quadrupole ICP-MS (Agilent Technologies). For ^{1}H relaxivity measurement, Bruker Minispec Mq20 (0.47 T, 20 MHz, 37 °C) was used. Bright-field and fluorescence images were obtained using an sCMOS camera FLOYD-200 (Wraymer Inc.) attached to an OLYMPUS polarization microscope BX51 with a liquid immersion objective lens (40 ×), an OLYMPUS U-MWU2 fluorescence mirror unit, and an excitation filter (330-385 nm). ImageJ software was used for processing and analysis of microscope images, [3]. For cytotoxicity assay using CCK-8, a Bio-Rad iMark Microplate Reader was used with a 96-well flat bottom microplate. Neutrons were irradiated using the Kyoto University Research Reactor.

1.3 Procedure

1.3.1 Preparation of 1 mM Ln_3TCAS_2 Solution:

To a 5 mL volumetric flask, 0.01 M $Ln(NO_3)_3$ solution (Ln: Gd or Tb) and a 0.01 M TCAS aqueous solution were added to achieve final concentrations of [Ln] = 3.0 mM and [TCAS] = 2.0 mM, respectively. To this mixture, 5.0 M ammonia solution was added to the mixture to adjust the pH to 10. The solution was then brought to volume with water and allowed to stand at 60 °C for 24 hours to afford 1.0 mM Ln_3TCAS_2 solution.

1.3.2 Preparation of PEI solutions

PEI (1–100 mg) was dissolved in ultrapure water to a volume (10.0 mL) to afford PEI solutions of 0.1–10.0 g/L. The pH was then adjusted to 7.0 by adding conc. HCl.

1.3.3 Preparation of Nanogel

While stirring 5.0 mL of the PEI solution at 2200 rpm, 1.0 mL of a 1.0 mM Ln_3TCAS_2 solution was added, and stirring was continued for 5 minutes. The mixture was then sonicated at 20 ± 2 °C for 30 minutes, followed by centrifugation at 10000 rpm for 20 minutes. After removing the supernatant, the precipitate was redispersed in 1.0 mL of ultrapure water to obtain the Ln_3TCAS_2–PEI nanogel solution.

1.3.4 Particle Size and Zeta Potential Measurements

The nanogel solution was diluted threefold and adjusted to match the composition of either 10 mM HEPES or PBS buffer. Particle size and ζ potential were then measured. The sample was stored at 37 °C and remeasured the following day. Particle size was reported as the average diameter corresponding to the peak of the intensity-weighted size distribution.

1.3.5 Weight Measurement of Nanogel

Weighing bottles were dried at 80 °C for 1 hour, cooled in a desiccator for 30 minutes, and weighed. This process was repeated three times to ensure a constant weight. The entire volume of the nanogel solution was transferred into the weighing bottle, and dried overnight in a desiccator together with a dish containing P_2O_5 powder. After drying, the same weighing process was repeated, and the difference from the empty bottle was recorded as the mass of the Ln_3TCAS_2–PEI nanogels.

1.3.6 Determination of Ln amount in Nanogel

The amount of Ln_3TCAS_2 loaded in the nanogels was determined using ICP-AES or ICP-MS. To the weighing bottle containing the dried nanogels, 2 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. The mixture was evaporated to dryness overnight at 95 °C. The residue was dissolved in 400 µL of 5 M nitric acid, diluted to 20 mL with ultrapure water in a volumetric flask, and further diluted to prepare the sample for analysis.

1.3.7 Calculation of Ln_3TCAS_2 Loading Efficiency and Loading Capacity

The loading efficiency (LE) and capacity (LC) were calculated using Eq. (1) and (2), respectively [4].

LE% = (Amount of Ln in nanogel [mol])/(Amount of Ln in the solution to prepare nanogel [mol])×100% (1)

LC% = (Weight of Ln_3TCAS_2 in nanogel [g])/(Weight of nanogel [g])×100% (2)

The Ln amount in the nanogel was determined using the procedure described above. The weight of the Ln_3TCAS_2 installed in the nanogel was calculated using the Ln amount.

1.3.8 Determination of Tb_3TCAS_2 Leakage Rate:

Tb_3TCAS_2–PEI nanogel solutions ($N = 3$) were diluted with PBS to [Tb] = 25 μM and incubated at 37 °C for 24 h. After incubation, a 6 mL aliquot was transferred to a centrifuge tube equipped with a 10,000 MWCO membrane and centrifuged at 7000 × g for 10 minutes. The resulting filtrate (4 mL) was transferred to a 5 mL volumetric flask, followed by the addition of 0.1 mL of concentrated nitric acid and deionized water to bring the volume to 5 mL. The terbium concentration in the solution was then quantified using ICP-AES. The leakage rate was calculated using Eq. (3).

Leakage rate = (Tb concentration in the supernatant)/(Initial Tb concentration in the nanogel solution)×100%, (3)

1.3.9 ^1H relaxivity measurement

After determining the Gd concentration of the Gd_3TCAS_2–PEI nanogel solution by ICP-AES, the solution was diluted to [Gd] = 1.0 mM in 10 mM HEPES solution (pH 7.4) to obtain a specimen. The solution was further diluted with 10 mM HEPES solution (pH 7.4) solution and subjected to the Bruker Minispec Mq20 to measure longitudinal (T_1) and transverse (T_2) relaxation times. For the Gd_3TCAS_2 complex, a stock solution of Gd_3TCAS_2 ([Gd] = 1.0 mM) was prepared in 10 mM HEPES (pH 7.4), which was diluted with 10 mM HEPES solution (pH 7.4) for T_1 and T_2 measurements. The inversion recovery and Carr-Purcell-Meiboom-Gill sequences were used to measure T_1 and T_2, respectively. The longitudinal and transverse relaxivities (r_i, i = 1 and 2, respectively) were estimated by fitting Eq. (S1) to the dependence of the relaxation rate ($R_i = 1/T_i$) on the total Gd concentration in the sample solutions.

$$R_i = R_{i,0} + r_i[\mathrm{Gd}] \quad (i = 1, 2). \tag{S1}$$

1.3.10 Cell Subculturing

The cells were cultured in an RPMI 1640 medium containing 10% FBS and 2.5% PS at 37 °C in a humidified air atmosphere containing 5% CO_2. Subculturing was conducted when the cells reached 70–80% confluency. For passaging, the medium in the culture flasks (25 cm^2 of adhesion area) was removed, and 5 mL of PBS was gently added and washed, followed by the removal of PBS three times. After 1 mL of trypsin/EDTA was added and the cells were incubated for 2 min, they were detached from the adhesive surface. Then, 4 mL of fresh medium was added and the entire volume was transferred to a centrifuge tube. After centrifugation at 4 °C and 1,500 rpm for 3 min, the supernatant was removed, and 1.2 mL of fresh medium was added. A new culture flask was prepared, and 5 mL fresh medium and cell suspension were added and incubated.

1.3.11 Cell counting

Cell counts were determined by injecting 10 μL of cell suspension into each chamber using a C-Chip Disposable Hemocytometer (NanoEnTek Inc.), observing with an inverted microscope, and counting the number of cells.

1.3.12 Fluorescent microscope imaging of the cells

In a culture dish with a bottom diameter of 35 mm, a cell suspension containing 1.0×10^5 cells/mL was inoculated in a volume of 2 mL and incubated overnight. After the incubation period, the culture medium was aspirated and the cells were subjected to a sequential double wash with 1 mL of PBS to eliminate the residual wash solution (unless stated otherwise, the cell washing protocol remained constant). Subsequently, a solution comprising the medium and Tb_3TCAS_2-loaded nanogel was combined to achieve a volumetric ratio of 9:1. This resulted in the formulation of the test solution ([Tb] = 25 μM), which was subsequently applied at 2 mL. Post a 24 h incubation, the supernatant was aspirated, and the cells were washed with PBS. After washing, 1 mL of PBS was added, and the cells were examined under a fluorescence microscope to acquire fluorescent images.

1.3.13 Determination of cellular uptake of Gd_3TCAS_2-loaded nanogel:

In a 6-well plate, a cell suspension with a concentration of 1.0×10^5 cells/mL was seeded at 2 mL and incubated overnight. Following incubation, the culture medium was removed and the cells were washed with PBS. Subsequently, a solution of the medium versus Gd_3TCAS_2-loaded nanogel solution with a volume ratio of 9:1 was prepared and added to 2 mL to obtain a test

solution ([Gd] = 12 and 25 μM). After a 24 h incubation, the supernatant was removed, and the cells were washed with PBS. After washing, 1 mL of trypsin/EDTA solution was added and the cells were incubated for 2 min to detach them from the adherent surface. Following detachment, 1 mL of medium was added to the plate, and the suspension was collected in a 15 mL centrifuge tube. This process was repeated twice, followed by centrifugation (4 °C, 1,500 rpm, 3 min). After centrifugation, the supernatant was completely removed, and 500 μL of PBS was added to the cell suspension. A portion of the collected cell suspension was used for cell counting, while the remainder was transferred to sample tubes, followed by the addition of 2 mL of concentrated nitric acid and 2 mL of hydrogen peroxide. The mixture was evaporated and dried overnight at 95 °C. The dried material was dissolved in 5 M nitric acid (100 μL), and the solution was diluted to volume with pure water using a 5 mL volumetric flask. The resulting measurement solutions were analyzed using ICP-AES. The cellular uptake rate of Gd was calculated using the following equation:

Cellular uptake rate (%) = (Amount of Gd introduced into cells [mol])/(Amount of Gd added to the medium [mol])×100 (4)

1.3.14 Cell Viability Measurement

A cell suspension with a concentration of 2.5×10^4 cells/mL was seeded in flat-bottom 96-well plates at 100 μL/well and incubated overnight. Subsequently, the culture medium was removed, and a test solution ([Gd] = 0–100 μM) with a medium to Gd_3TCAS_2-loaded nanogel solution volume ratio of 9:1 was mixed and added at 100 μL/well. After a 24 h incubation, CCK-8 solution was introduced at 10 μL/well, followed by a 3 h colorimetric reaction at room temperature. The absorbance of each well was measured using a microplate reader at 450 nm. The cell survival rate was calculated using Eq. (S2) below, where A_C, A_0, and A_b represent the absorbance under conditions of Gd concentration C μM, 0 μM, and PBS-only background, respectively.

$$\text{Cell Viability} = (A_C - A_b)/(A_0 - A_b) \times 100\% \quad \text{(S2)}$$

1.3.15 Viability Assessment

PBS (5 mL), Calcein-AM stock solution in DMSO (10 μL), and PI stock solution in water (15 μL) were combined in a 15 mL centrifuge tube to create a staining solution for live and dead

cells. Cell seeding was carried out following the procedure outlined in the "*1.3.12 Fluorescent microscope imaging of the cells*." The Gd_3TCAS_2–PEI nanogel solution was added to the medium to achieve a concentration [Gd] = 0–100 μM. After a 24 h incubation, the cells were washed with PBS and 1 mL of the staining reagent was added. Following a 30 min incubation, the cells were washed with PBS, and 1 mL of PBS was added. Fluorescence microscopy was used to observe cells and capture fluorescence images.

1.3.16 Investigation of Cellular Uptake Pathways of Gd_3TCAS_2–PEI nanogel

(1) Endocytosis Inhibition: Following the protocol outlined in "*1.3.12 Fluorescent microscope imaging of the cells*," cell seeding was performed. A test solution ([Tb] = 25 μM) with a medium to Tb_3TCAS_2–PEI nanogel volume ratio of 9:1 was added at 2 mL. After a 4 h incubation at 4 °C, cells were washed with PBS. Subsequently, 1 mL of PBS was added, and the cells were observed under a fluorescence microscope to capture fluorescence images.

(2) Clathrin-Mediated Endocytosis Inhibition [5]: A cell suspension with a concentration of 1.0×10^5 cells/mL was seeded in a 35 mm culture dish at a volume of 2 mL and incubated overnight. After incubation, the culture medium was aspirated, and the cells were washed with PBS. Next, 2 mL of an inhibition solution with a medium to sucrose solution volume ratio of 8:2 ([Sucrose] = 0.45 M) was added. After a 0.5 h incubation, the supernatant was removed, and the cells were washed with PBS. Subsequently, a test solution ([Tb] = 25 μM) with a medium to Tb_3TCAS_2–PEI nanogel volume ratio of 9:1 was added at 2 mL. Following a 2 h incubation, the supernatant was removed, and the cells were washed with PBS. After washing, 1 mL of PBS was added, and the cells were observed under a fluorescence microscope to capture fluorescent images.

(3) Caveola-Mediated Endocytosis Inhibition Experiment [6]: A cell suspension with a concentration of 1.0×10^5 cells/mL was seeded in a 35 mm culture dish and incubated overnight. After incubation, the culture medium was aspirated, and the cells were washed with PBS. Following this, an inhibition solution with a medium to methyl-β-cyclodextrin (MβCD) solution volume ratio of 9:1 ([MβCD] = 10 mM) was added at 2 mL. After a 1 h incubation, the supernatant was removed, and the cells were washed with PBS. Subsequently, a test solution ([Tb] = 25 μM) with a medium to Tb_3TCAS_2–PEI nanogel volume ratio of 9:1 was added at 2 mL. After a 2 h incubation, the supernatant was removed, and the cells were washed with PBS. After washing, 1 mL of PBS was added, and the cells were observed under a fluorescence microscope to capture fluorescent images.

1.3.17 Investigation of Intracellular Distribution

Cells were seeded and cultured overnight following the same protocol used for fluorescence imaging of Tb_3TCAS_2–PEI nanogels. After incubation, the culture medium was replaced with fresh medium containing Tb_3TCAS_2–PEI nanogel ([Tb] = 25 μM), and the cells were incubated for 24 hours. The lysosome staining solution was prepared by diluting LysoPrime Green 1:2000 in MSF buffer (5.4 mM KCl, 137 mM NaCl, 8.3 mM glucose, 0.44 mM KH_2PO_4, 0.33 Na_2HPO_4, 10 mM HEPES, 1 mM $MgCl_6$, 1 mM $CaCl_2$) [7]. The nuclear staining solution was prepared by diluting a 1 mg/mL stock solution of Hoechst 33342 in PBS to a final concentration of 1 μg/mL. Subsequently, the culture medium was removed from the dish, and the cells were washed with PBS. Lysosome staining solution (1 mL) was then added, followed by incubation at 37 °C for 30 minutes. After removal of the staining solution and PBS washing, 1 mL of nuclear staining solution was added, and the cells were left to stand in the dark at room temperature for 5 minutes. Finally, the staining solution was removed, the cells were washed with PBS, and 1 mL of PBS was added before fluorescence microscopy was performed to acquire fluorescence images.

1.3.18 Neutron Irradiation Experiment

We followed the protocol recommended for *in vitro* boron NCT effect described in the literature [8]. In a 6-well plate, a cell suspension with a concentration of 1.0×10^5 cells/mL was seeded at 2 mL/well and incubated overnight. Following incubation, the culture medium was removed, and the cells were washed with PBS. Subsequently, a solution of medium versus Gd-agent solution, with a volume ratio of 9:1, was prepared and added at 2 mL to achieve a test solution ([Gd] = 12 or 25 μM). Gd agents, including Gd_3TCAS_2-loaded nanogel, Gd_3TCAS_2 complex, Gd-DTPA complex, and PBS (control), were utilized. After a 24 h incubation, the supernatant was removed, and the cells were washed with PBS. After washing, 1 mL of trypsin/EDTA solution was added and the cells were incubated for 2 min to detach them from the adherent surface. Following detachment, 1 mL of medium was added to the plate, and the suspension was collected in a 15 mL centrifuge tube. This process was repeated twice, followed by centrifugation (4 °C, 1,500 rpm, 3 min). After centrifugation, the supernatant was completely removed, and PBS (500 μL) was added to the cell suspension. The collected cell suspension was diluted to a concentration of 2.0×10^4 cells/mL and 1 mL aliquot was transferred to four 2 mL cryotubes. Cryotubes were arranged on a PTFE table (Fig. S1) [8], and were irradiated by thermal and epithermal neutrons using Kyoto University Research Reactor (1 MW, fluence:

2.09×10^{11}–2.06×10^{12} thermal neutrons cm^{-2}, 3.72×10^{10}–3.67×10^{11} epithermal neutrons cm^{-2}) for a duration of 20 min (Table S1). Samples with varying neutron doses were placed upstream, midstream, and downstream (Fig. S1). In addition, each irradiation session involved exposure of two samples. The neutron dose was determined based on the radioactivity of the gold foils affixed to the cryotube along the direction of the incidence of thermal neutrons [8]. γ-Rays that are not intended for GdNCT were determined by thermoluminescent dosimeters (TLDs) placed on the PTFE table (Table S1). The cell viability was estimated using a colony assay.

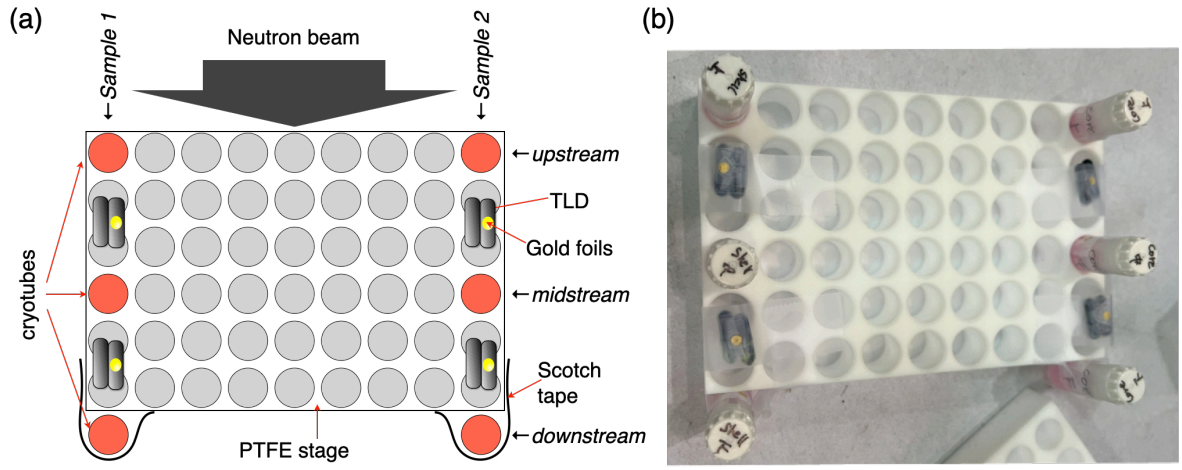

Fig. S1 (a) Illustration and (b) photograph of the sample setup for neutron irradiation (top view).

Table S1 Neutron fluence and physical dose. [a)]

Sample	position	Au foil	Fluence / cm^{-2}		Physical Dose / Gy				
			Therm. n	Epi. n	Therm. n	Epi. n	Fast n	γ ray	Sum
Nanogel	up	front	1.92E+12	3.41E+11	2.56E-01	2.73E-02	1.90E-01	2.51E-01	7.24E-01
		rear	8.00E+11	1.42E+11	1.07E-01	1.14E-02	7.90E-02	2.51E-01	4.48E-01
	mid	front	1.29E+12	2.30E+11	1.72E-01	1.83E-02	1.28E-01	2.51E-01	5.69E-01
		rear	5.79E+11	1.03E+11	7.71E-02	8.22E-03	5.72E-02	1.57E-01	3.00E-01
	down	front	7.11E+11	1.26E+11	9.48E-02	1.01E-02	7.02E-02	1.57E-01	3.32E-01
		rear	2.43E+11	4.32E+10	3.24E-02	3.45E-03	2.40E-02	1.57E-01	2.17E-01
Gd_3TCAS_2	up	front	2.06E+12	3.67E+11	2.75E-01	2.93E-02	2.04E-01	2.58E-01	7.67E-01
		rear	9.47E+11	1.68E+11	1.26E-01	1.35E-02	9.36E-02	2.58E-01	4.92E-01
	mid	front	1.04E+12	1.85E+11	1.39E-01	1.48E-02	1.03E-01	2.58E-01	5.14E-01
		rear	4.88E+11	8.68E+10	6.50E-02	6.93E-03	4.82E-02	1.55E-01	2.75E-01
	down	front	7.33E+11	1.30E+11	9.77E-02	1.04E-02	7.24E-02	1.55E-01	3.36E-01
		rear	2.32E+11	4.12E+10	3.09E-02	3.29E-03	2.29E-02	1.55E-01	2.12E-01
Gd-DTPA	up	front	2.03E+12	3.62E+11	2.71E-01	2.89E-02	2.01E-01	1.73E-01	6.74E-01
		rear	7.97E+11	1.42E+11	1.06E-01	1.13E-02	7.88E-02	1.73E-01	3.70E-01
	mid	front	1.24E+12	2.20E+11	1.65E-01	1.76E-02	1.22E-01	1.73E-01	4.78E-01
		rear	4.66E+11	8.29E+10	6.21E-02	6.62E-03	4.60E-02	1.79E-01	2.93E-01
	down	front	7.34E+11	1.31E+11	9.79E-02	1.04E-02	7.25E-02	1.79E-01	3.59E-01
		rear	2.11E+11	3.75E+10	2.81E-02	2.99E-03	2.08E-02	1.79E-01	2.30E-01
Control	up	front	2.02E+12	3.60E+11	2.70E-01	2.87E-02	2.00E-01	2.18E-01	7.16E-01
		rear	9.27E+11	1.65E+11	1.24E-01	1.32E-02	9.16E-02	2.18E-01	4.46E-01
	mid	front	1.26E+12	2.23E+11	1.67E-01	1.78E-02	1.24E-01	2.18E-01	5.27E-01
		rear	4.75E+11	8.45E+10	6.34E-02	6.75E-03	4.70E-02	2.07E-01	3.24E-01
	down	front	7.36E+11	1.31E+11	9.81E-02	1.05E-02	7.27E-02	2.07E-01	3.88E-01
		rear	2.09E+11	3.72E+10	2.79E-02	2.97E-03	2.07E-02	2.07E-01	2.58E-01

a) Therm. n: thermal neutron, Epi. n: epithermal neutron, Fast n: fast neutron.

1.3.19 Colony Assay

After irradiation, the cell suspensions were seeded at 10 μL/well in 6-well plates containing 2 mL of medium per well. The plates were then incubated at 37 °C for 14 d. Subsequently, the culture medium was removed, and the cells were washed with PBS. Ethanol (1 mL/well) was added, and the cells were fixed by allowing them to stand for 5 min before aspirating the solution. Crystal violet (500 μL/well) was added, left to stand for 20 min, and removed. The cells were washed with distilled water, air-dried, and individual colonies in each well were counted using ImageJ software [3]. The cell viability was estimated by dividing the number of colonies by the number of colonies in a sample with no neutron irradiation.

2. Estimation of the charges of PEI and Ln_3TCAS_2 in the nanogel formation step

The concentrations of charges on PEI and the Ln_3TCAS_2 complex during gel preparation were estimated as follows (Ln = Tb):

I. The repeating unit of PEI was assumed to be "-($NHCH_2CH_2NHCH_2CH_2N(CH_2CH_2NH_2)CH_2CH_2$)-," (Fig. 1) based on prior reports indicating a ratio of one primary, two secondary, and one tertiary amine in a branched PEI [9]. It was also assumed that the pK_a values of these amines are independent of the degree of protonation of PEI. The pK_a values of the protonated PEI amines used were 4.5 (primary), 6.7 (secondary), and 11.6 (tertiary) according to the literature [10].

II. The concentration of positive charges contributed by protonated PEI amines was estimated as follows (Table S2):
i) First, the molar concentrations of primary, secondary, and tertiary amines were calculated from the PEI concentration (g/L) used during gel preparation, based on the molecular weight (168 Da) of the repeating unit and the number of amines per unit.
ii) Next, using the pH of the gel solution and the respective pK_a values, the fractions of protonated amines were determined.
iii) Multiplying these values yielded the concentrations of protonated amines, and their sum gave the total concentration of positive charges from PEI.

III. The concentration of negative charges from Ln_3TCAS_2 was calculated by multiplying its molar concentration by 7, corresponding to the complex's net charge.

IV. Finally, the net charge was determined by subtracting the total negative charge from the total positive charge.

The results indicated that the positive charge, predominantly contributed by protonated tertiary amines, consistently exceeded the negative charge from Ln_3TCAS_2.

Table S2 Estimation of charges arising from the PEI and Tb_3TCAS_2 components in the nanogel preparation step

Experimental Conditions	PEI solution used / g L^{-1}						
	0.1	0.5	1	2	2.5	5	10
pH of the gel solution	9.9*	9.85	9.47	8.87	8.64	8.13	7.68
error (n = 3)		±0.01	±0.06	±0.13	±0.08	±0.09	0.04
PEI							
Concn. in the gel preparation step / g L^{-1}	0.08	0.42	0.83	1.67	2.08	4.17	8.33
i) Concn. of amino groups / M							
primary	4.96E-04	2.48E-03	4.96E-03	9.92E-03	1.24E-02	2.48E-02	4.96E-02
secondary	9.92E-04	4.96E-03	9.92E-03	1.98E-02	2.48E-02	4.96E-02	9.92E-02
tertiary	4.96E-04	2.48E-03	4.96E-03	9.92E-03	1.24E-02	2.48E-02	4.96E-02
ii) Mol fraction of protonated NH^+							
primary	0.000	0.000	0.000	0.000	0.000	0.000	0.001
secondary	0.001	0.001	0.002	0.007	0.011	0.036	0.095
tertiary	0.980	0.983	0.993	0.998	0.999	1.000	1.000
iii) Concn. of NH^+ / M							
primary	2.0E-09	1.1E-08	5.3E-08	4.2E-07	9.0E-07	5.8E-06	3.3E-05
secondary	6.3E-07	3.5E-06	1.7E-05	1.3E-04	2.8E-04	1.8E-03	9.4E-03
tertiary	4.9E-04	2.4E-03	4.9E-03	9.9E-03	1.2E-02	2.5E-02	5.0E-02
Total positive charge / M	4.9E-04	2.4E-03	4.9E-03	1.0E-02	1.3E-02	2.7E-02	5.9E-02
Tb_3TCAS_2							
Concn. in the gel preparation step / M	1.7E-04	1.7E-04	1.7E-04	1.7E-04	1.7E-04	1.7E-04	1.7E-04
Total negative charge / M	1.2E-03	1.2E-03	1.2E-03	1.2E-03	1.2E-03	1.2E-03	1.2E-03
Net charge / M							
Positive – Negative	-6.8E-04	1.3E-03	3.8E-03	8.9E-03	1.2E-02	2.5E-02	5.8E-02

*Not measured, but estimated by the PEI 0.5 (pH 9.85) and PEI 0 (i.e. Tb_3TCAS_2 solution of pH 10.0).

3. ^{1}H relaxivity

Longitudinal and transverse relaxation rates, $R_i = 1/T_i$ (i = 1, 2), were measured for Gd_3TCAS_2–PEI nanogels and Gd_3TCAS_2 complexes over a range of Gd concentrations ([Gd] = 0–0.5 mM) in 10 mM HEPES buffer (pH 7.4) at 37 °C (Figs. 3A and S2). Relaxivities (r_1, r_2) were obtained by fitting Eq. (S1) to the R_i data (Table S3, Fig. 3B).

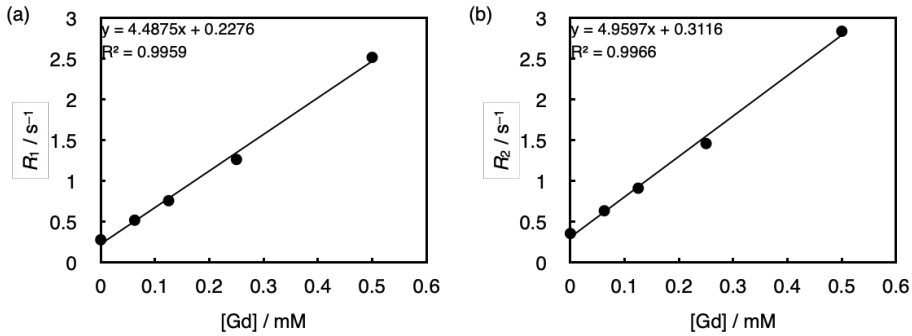

Fig. S2 Dependence of relaxation rates R_i (i = 1, 2) of water ^{1}H for Gd_3TCAS_2 solutions on the Gd concentration measured in 10 mM HEPES at 0.47 T, 20 MHz, 37°C.

Table S3 Relaxivity of Gd_3TCAS_2–PEI nanogels and Gd agents (0.47 T, 37°C).

Agents	r_1 / mM^{-1} s^{-1}	r_2 / mM^{-1} s^{-1}	Ref.
Gd_3TCAS_2–PEI nanogel	8.84	12.7	This work
Gd_3TCAS_2	4.49	4.96	This work
Gd_3TCAS_2-ANP	10.7	15.4	[11]
Gd-DTPA	3.4	4.0	[12]
Gd-DOTA	3.4	4.1	[12]

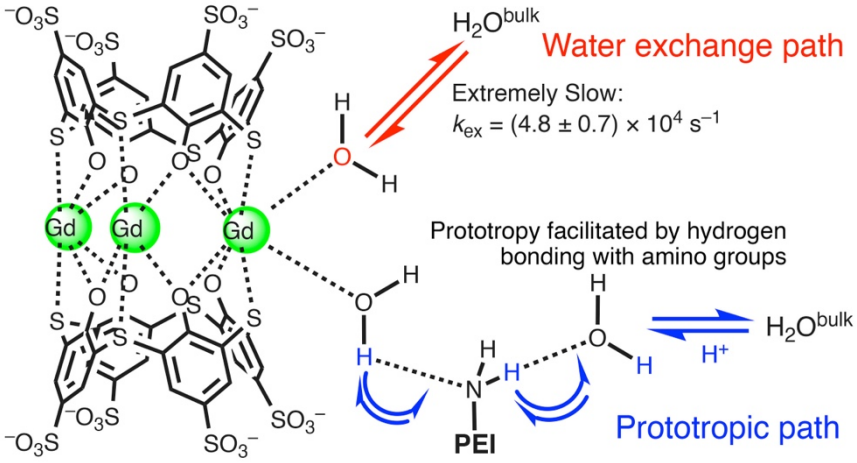

Fig. S3 Water exchange and prototropic relaxation path.

4. Time lapse of cellular uptake

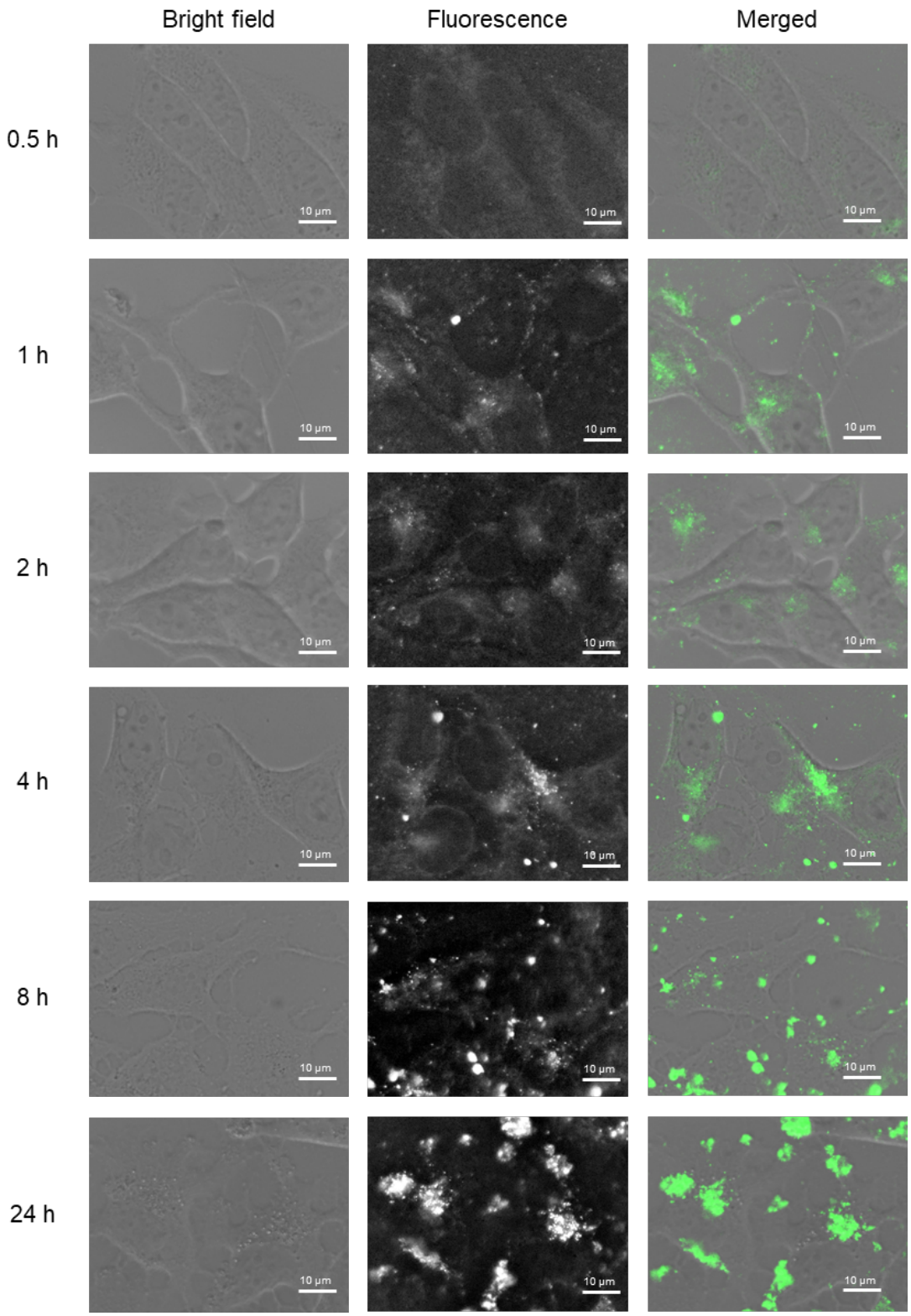

Fig. S4 Bright field and fluorescence images of MCF-7 cells incubated in the presence of Tb_3TCAS_2–PEI nanogels (PEI 0.5 g/L) for different incubation times (0.5–24 h). [Tb] = 25 μM.

5. Inhibition studies on cellular uptake path

To elucidate the internalization pathway of Gd_3TCAS_2–nanogels, an inhibition study was conducted. It is well-established that nanomaterials are typically internalized via endocytic pathways following interactions with the cell membrane [13]. When MCF-7 cells were incubated with Tb_3TCAS_2–nanogels ([Tb] = 25 μM) at a low temperature (4 °C) for 2 h, a marked reduction in fluorescence intensity was observed compared to the condition at 37 °C (Fig. S5), indicating that nanogels were uptaken by energy-dependent endocytosis pathways.

To investigate whether the uptake occurs via caveolae-mediated or clathrin-mediated endocytosis, the cells were pre-treated with methyl-β-cyclodextrin (MβCD), a known inhibitor of caveolae-mediated endocytosis [6], or sucrose, an inhibitor of clathrin-mediated endocytosis [5]. After inhibitor treatment, the cells were incubated with Tb_3TCAS_2–nanogels for 2 h at 37 °C (Fig. S6). In both cases, a noticeable decrease in green luminescence was observed compared to untreated cells. Although the inhibition was incomplete, the results suggest that both caveolae- and clathrin-mediated pathways are involved in the cellular uptake of the nanogel.

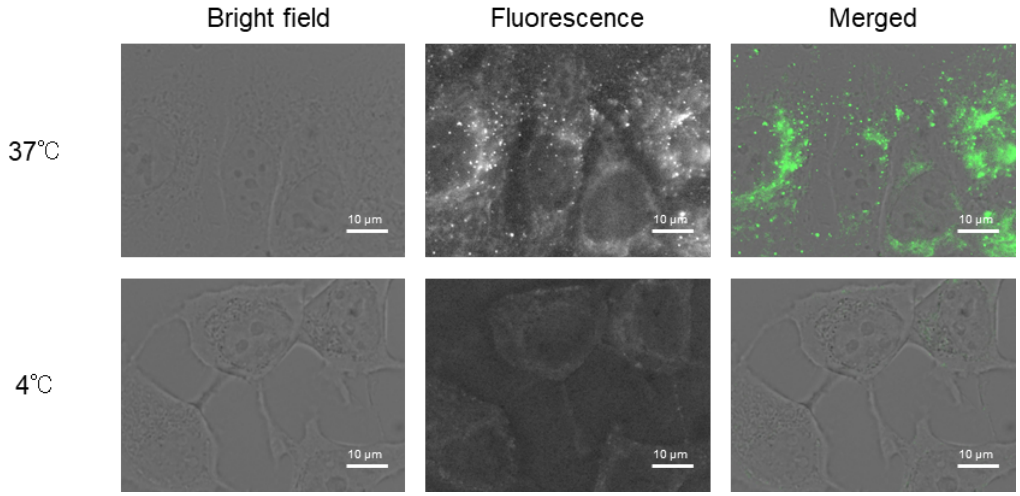

Fig. S5 Bright field and fluorescence images of MCF-7 cells incubated for 2 h in the presence of Tb_3TCAS_2–PEI nanogels (PEI 0.5 g/L). Incubation at 37 and 4℃ for 2 h in the presence of Tb_3TCAS_2–PEI nanogels ([Tb] = 25 μM).

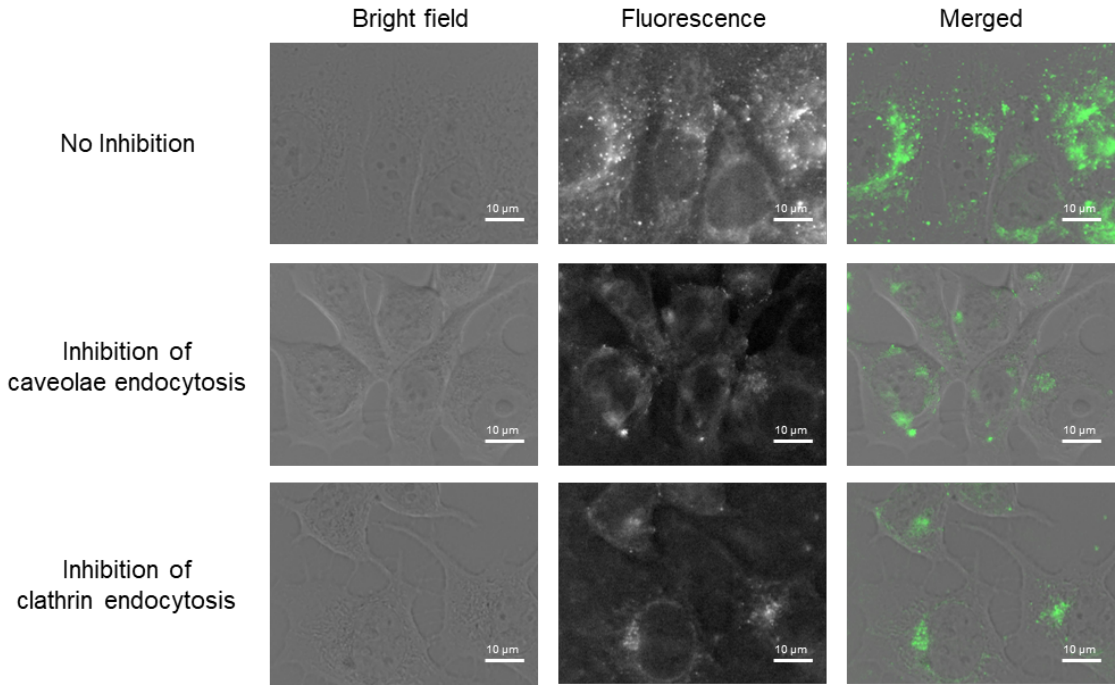

Fig. S6 Brightfield and fluorescence images of MCF-7 cells incubated in the presence of Tb_3TCAS_2–PEI nanogel (PEI 0.5 g/L) for 2 hours. Middle row: incubated with 10 mM Methyl-β-cyclodextrin for 1 hour at 37°C, lower row: incubated with 0.45 M Sucrose for 30 minutes at 37°C. After inhibition, incubated with Tb_3TCAS_2–PEI nanogel ([Tb] = 25 µM) for 2 hours at 37°C.

6. Analysis of Subcellular Localization

In Fig. S7a, the fluorescence images of the nucleus-staining dye Hoechst 33342 and Tb_3TCAS_2 were merged into a single image and then overlaid with the fluorescence image of LysoPrime Green, a lysosome-staining dye. This approach was adopted because both Hoechst 33342 and Tb_3TCAS_2 are observed under the same UV excitation filter. In the merged image, Hoechst 33342 and Tb_3TCAS_2 were displayed in blue, while LysoPrime Green was shown in red. As a result, partial overlap between the blue and red signals appeared as purple regions, suggesting that the Ln_3TCAS_2–PEI nanogel may be distributed within lysosomes. However, this method assumes that no nanogels are localized in the nucleus, which is a potential limitation.

To address this, in Fig. S7b, the individual fluorescence components of Hoechst 33342 and Tb_3TCAS_2 were separated and independently processed before overlaying. In this corrected composite image, regions where the green luminescence of Tb_3TCAS_2 and the red fluorescence of LysoPrime Green overlapped appeared yellow, while no significant overlap between the green fluorescence of Tb_3TCAS_2 and the blue fluorescence of Hoechst 33342 was observed. These findings support that the Ln_3TCAS_2–PEI nanogels are predominantly localized in lysosomes. This conclusion is consistent with the results of the inhibitory study (Section 6), which indicated that the nanogels are internalized via endocytosis.

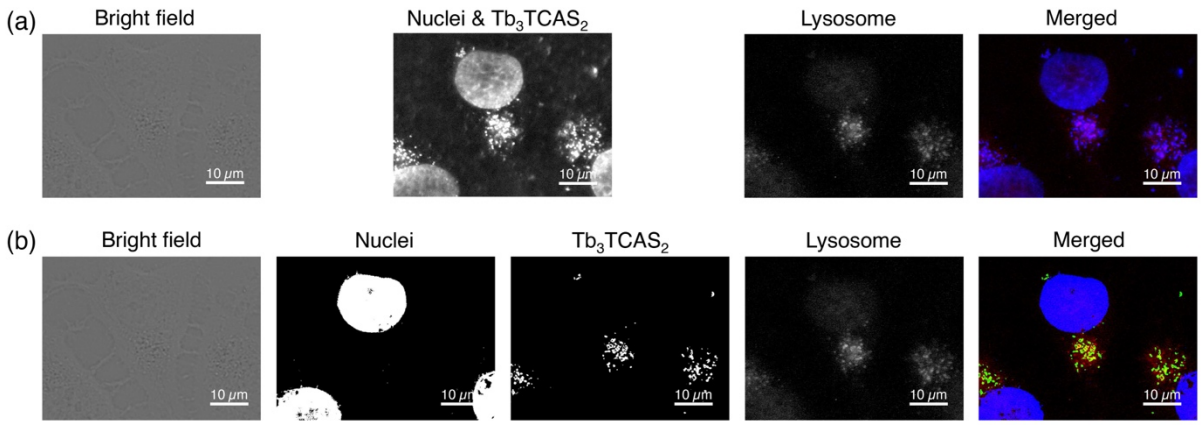

Fig. S7 Subcellular localization of Tb_3TCAS_2–PEI nanogels in MCF-7 cells. The nuclei were stained with Hoechst 33342, and lysosomes were stained with LysoPrime Green. (a) Fluorescence images of Hoechst 33342 and Tb_3TCAS_2 were combined into a single channel and overlaid with the fluorescence image of LysoPrime Green. Hoechst 33342 and Tb_3TCAS_2: blue; lysosomes: red. (b) Fluorescence signals of Hoechst 33342 and Tb_3TCAS_2 were separated by extracting individual color components from their merged image and then overlaid with the fluorescence image of LysoPrime Green. Hoechst 33342: blue; Tb_3TCAS_2: green; lysosomes: red.

7. Cytotoxicity

As shown in Fig. 5B, the cell viability data was fitted with the four-parameter logistic curve (eq. S3).

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{c}\right)^{b}} \quad \text{(S3)}$$

Here, max and min represent the upper and lower asymptotes, respectively; c denotes the x-value at the midpoint of the curve where y = (max + min)/2, corresponding to the CC_{50} (in μM); and b is the slope parameter that describes the steepness of the curve. For the fitting max and min were fixed to 100 and 0, respectively. The obtained b and c were 2.63 and 47.4 μM, respectively

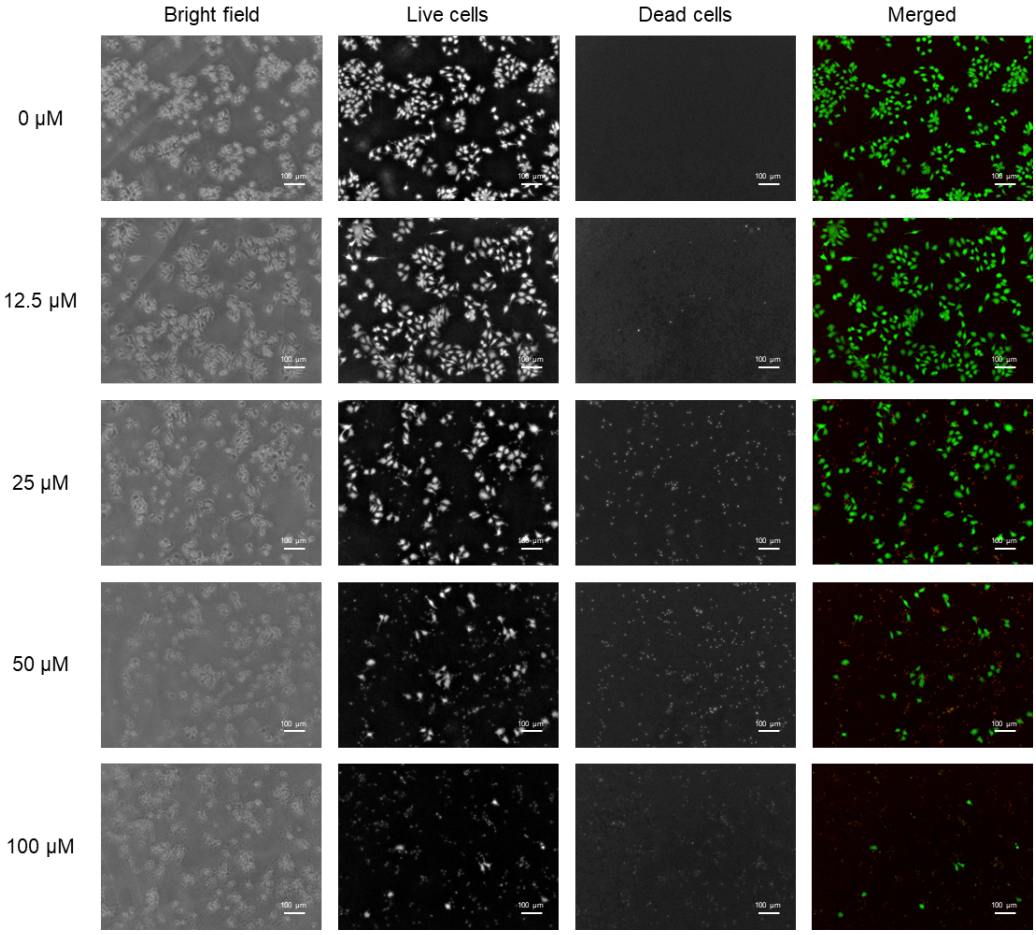

Fig. S8 Bright field and fluorescence images of live and dead MCF-7 cells incubated with Gd_3TCAS_2–PEI nanogels at different Gd concentrations for 24 h.

8. NCT efficacy

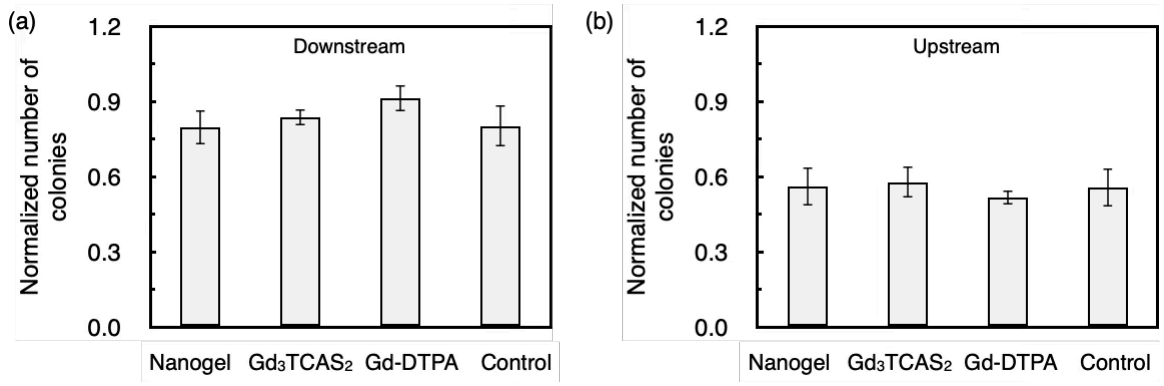

Fig. S9 Cell viability was assessed based on the number of colonies, normalized to that of the non-irradiated control sample. MCF-7 cells were incubated with Gd agents ([Gd] = 12 μM), followed by removal of unbound Gd agents and subsequent irradiation with thermal neutrons at the (a) downstream and (b) upstream positions. Neutron fluence is given in Table S1. "Control" refers to cells incubated without Gd agents.

Table S4 Comparison of the NCT effect on cell viability using Gd_3TCAS_2-loaded ANP and PEI nanogel. [a)]

	ANP (Ref. 13)	PEI nanogel (This work)
Viability of Control	71.7 ± 3.3%	73.2 ± 3.8%
Viability in the presence of Gd agent	56.9 ± 6.5%	63.6 ± 2.4%
Gd concentration during incubation / μM	50	12

a) All other experimental conditions, including the cell line (MCF-7), incubation time, and neutron irradiation setup, were consistent between the two experiments.

References

[1] N. Iki, T. Horiuchi, H. Oka, K. Koyama, N. Morohashi, C. Kabuto, S. Miyano, Energy transfer luminescence of Tb3+ ion complexed with calix[4]arenetetrasulfonate and the thia and sulfonyl analogue. The effect of bridging groups, Journal of the Chemical Society, Perkin Transactions 2 (11) (2001) 2219–2225. 10.1039/B009151K

[2] K. Ueno, Chelatometry, Nankodo, Tokyo, 1989.

[3] W.S. Rasband, ImageJ, 1997–2018. https://imagej.net/ij/.

[4] Z. Shen, W. Wei, Y. Zhao, G. Ma, T. Dobashi, Y. Maki, Z. Su, J. Wan, Thermosensitive polymer-conjugated albumin nanospheres as thermal targeting anti-cancer drug carrier, European Journal of Pharmaceutical Sciences 35(4) (2008) 271–282. https://doi.org/10.1016/j.ejps.2008.07.006

[5] R.K. Khurana, R. Kumar, B.L. Gaspar, G. Welsby, P. Welsby, P. Kesharwani, O.P. Katare, K.K. Singh, B. Singh, Clathrin-mediated endocytic uptake of PUFA enriched self-nanoemulsifying lipidic systems (SNELS) of an anticancer drug against triple negative cancer and DMBA induced preclinical tumor model, Materials Science and Engineering: C 91 (2018) 645–658. https://doi.org/10.1016/j.msec.2018.05.010

[6] Y.-L. Li, N. Van Cuong, M.-F. Hsieh, Endocytosis Pathways of the Folate Tethered Star-Shaped PEG-PCL Micelles in Cancer Cell Lines, Polymers, 2014, pp. 634–650. 10.3390/polym6030634

[7] H. Kurokawa, A. Taninaka, T. Yoshitomi, H. Shigekawa, H. Matsui, Near-Infrared Light Irradiation of Porphyrin-Modified Gold Nanoparticles Promotes Cancer-Cell-Specific Cytotoxicity, Molecules, 2022. 10.3390/molecules27041238

[8] Y. Hattori, T. Andoh, S. Kawabata, N. Hu, H. Michiue, H. Nakamura, T. Nomoto, M. Suzuki, T. Takata, H. Tanaka, T. Watanabe, K. Ono, Proposal of recommended experimental protocols for in vitro and in vivo evaluation methods of boron agents for neutron capture therapy, Journal of Radiation Research 64(6) (2023) 859–869. 10.1093/jrr/rrad064

[9] V. Paola, G. Antonella, O. Giancarlo, M. Andrea, Polyethylenimine In Medicinal Chemistry, Current Medicinal Chemistry 15(27) (2008) 2826–2839. http://dx.doi.org/10.2174/092986708786242778

[10] K.D. Demadis, M. Paspalaki, J. Theodorou, Controlled Release of Bis(phosphonate) Pharmaceuticals from Cationic Biodegradable Polymeric Matrices, Industrial & Engineering Chemistry Research 50(9) (2011) 5873–5876. 10.1021/ie102546g

[11] K. Ohama, M. Komiya, T. Yamatoya, R. Sawamura, R. Karashimada, S. Gao, Y. Ozawa, K. Osada, I. Aoki, T. Nagasaki, M. Suzuki, N. Iki, Albumin nanoparticle electrostatically loaded with highly anionic Gd-thiacalixarene complex for MRI-guided neutron capture therapy, Colloids and Surfaces A: Physicochemical and Engineering Aspects 699 (2024) 134579. https://doi.org/10.1016/j.colsurfa.2024.134579

[12] M. Rohrer, H. Bauer, J. Mintorovitch, M. Requardt, H.-J. Weinmann, Comparison of Magnetic Properties of MRI Contrast Media Solutions at Different Magnetic Field Strengths, Investigative Radiology 40(11) (2005).

[13] N. Oh, J.H. Park, Endocytosis and exocytosis of nanoparticles in mammalian cells, Int J Nanomedicine. 2014(9(Supplement 1)) (2014) 51–63. 10.2147/IJN.S26592